FEBS Letters 585 (2011) 1121–1126

journal homepage: www.FEBSLetters.org

Ubiquitin C-terminal hydrolase-L3 regulates Smad1 ubiquitination and osteoblast differentiation

Ji Young Kim [a], Jae-Mok Lee [b], Je-Yoel Cho [a,*]

[a] Department of Biochemistry, School of Dentistry, Kyungpook National University, Daegu, South Korea
[b] Department of Periodontolgy, School of Dentistry, Kyungpook National University, Daegu, South Korea

ARTICLE INFO

Article history:
Received 5 January 2011
Revised 18 March 2011
Accepted 23 March 2011
Available online 29 March 2011

Edited by Zhijie Chang

Keywords:
Uch-L3
Smad1
Deubiquitination
Osteoblast differentiation

ABSTRACT

Ubiquitin C-terminal hydrolase-L3 (Uch-L3), a deubiquitinating enzyme, is upregulated in bone morphogenetic protein 2-induced osteoblast differentiation. The mechanism and role of Uch-L3 in the process of osteoblast differentiation is unknown. We found that Uch-L3 physically interacts with Smad1 and dramatically decreases the amount of poly-ubiquitinated Smad1. Osteoblast differentiation was enhanced in the C2C12 cells stably transfected with Uch-L3. Otherwise, the siRNA knock-down of Uch-L3 resulted in the decrease of osteoblast differentiation. These results suggest that Uch-L3 enhances osteoblast differentiation through the stabilization of Smad1 signaling. Thus, Uch-L3 acts to fine-tune the process of Smad1 activation.

Structured summary of protein interactions:
UCHL3 physically interacts with **SMAD1** by anti tag coimmunoprecipitation (View interaction)
UCHL3 physically interacts with **SMAD1** by anti bait coimmunoprecipitation (View interaction)
SMAD1 physically interacts with **UCHL3** by anti tag coimmunoprecipitation (View interaction)
UCHL3 physically interacts with **SMAD1** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

The process of ubiquitination/deubiquitination is a pivotal mechanism of controlling protein action in various biological processes and diseases [1–5]. The loss of function in ubiquitin–proteasome system-related proteins results in the development of various diseases including neurodegenerative disease, cancers, and osteoporosis [1,2,6–8]. Deubiquitination, the removal of covalently attached ubiquitin from the target protein, is catalyzed by deubiquitinating enzymes to regulate the abundance and/or functional activity of target proteins. These enzymes consist of four groups, ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), otubain domain-containing proteins (OTUs), and Machado-Joseph domain (Josephin domain)-containing proteins (MJD) [9]. The UCHs family includes Uch-L1, Uch-L3, Uch-L5, and BAP1. Uch-L3 is expressed in various tissues [10]. The Uch-L3 gene is located on mouse chromosome 14 (human chromosome 13).

Bone morphogenetic proteins (BMPs) play important roles in skeletal development and bone formation. In vivo bone formation is induced by recombinant human BMP2 and BMP7 [11]. BMP2 and BMP7 initiate signal transduction by binding to BMP receptor type I and II, and cause the phosphorylation and activation of Smad 1/5/8. The activated Smads heterodimerize with Smad4 and translocate from the cytosol to the nucleus leading to expression of the down-stream target genes. In TGFβ signaling, two known deubiquitinating (DUB) enzymes, Usp9X (FAM) and Uch-L5 (Uch37) are involved in the stability and activation of target proteins [12,13]. In Wnt/β-catenin signaling, Uch-L1 positively regulates the process by the increased stability of β-catenin [14]. The regulation of ubiquitination and/or deubiquitination is also critical in the regulation of cellular metabolism [15–17].

The loss of Uch-L3 expression showed retinal degeneration, skeletal muscle degeneration, and weakness of testis following cryptorchid injury [18–20]. Two recent reports suggest a function for Uch-L3 in obesity and insulin signaling [21,22]. Furthermore, the induction of the stress response was observed in the mouse embryonic fibroblast (MEF) cells from Uch-L3 deficient mice; the MEF cells showed up-regulation of cleaved ATF6, Grp78, and some HSPs and the accumulation of poly-ubiquitinated proteins [23]. It suggests the defective role of Uch-L3's deubiquitinating activity might cause the increase of poly-ubiquitination on target proteins. Uch-L3 was also shown to be involved in oncogenesis [24–26].

* Corresponding author. Address: Department of Biochemistry, School of Dentistry, Kyungpook National University, 101 Dongin-dong 2-ga, Daegu 700-422, South Korea. Fax: +82 53 421 1418.
E-mail address: jeycho@knu.ac.kr (J.-Y. Cho).

0014-5793/$36.00 © 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2011.03.053

In our recent proteomic study, Uch-L3 was found to be a regulator for identifying the specific targets in bone morphogenetic protein (BMP) 2-induced osteoblast differentiation [27]. There is limited information on the function of Uch-L3 in bone metabolism. Therefore, the current study focuses on the function of Uch-L3 in BMP2-induced osteoblast differentiation.

2. Materials and methods

2.1. Cells, reagents and plasmids

The mouse premyoblast C2C12 cells and the human embryonic kidney HEK293 cells were maintained as previously described [28,29]. The primary osteoblast cells were isolated from mouse long bone according to the previous reports [30,31]. The construct of pCMV2-Flag-tagged Smad1 and pCI-neo-Flag-Uch-L3 were gifts from Dr. T. Katagiri (Department of Biochemistry, School of Dentistry, Showa University, Tokyo, Japan) and Dr. K. Wada (Department of Degenerative Neurological Diseases, National Institute of Neuroscience, Tokyo, Japan), respectively. The retroviral expression vector for Uch-L3 plasmid 22564 and pCMV5-HA-Smad1 plasmid 14956 were purchased from Addgene [32,33]. Anti-Uch-L3, anti-Samd1, anti-phospho-Smad 1/5/8 antibodies were from Cell Signaling (MA, USA) and anti-HA, anti-Flag, anti-β-actin antibodies, and alkaline phosphatase staining kit were from Sigma–Aldrich (MO, USA).

2.2. RNA isolation and real-time RT-PCR

The total RNA was extracted by TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The real-time PCR was performed with the DNA Engine OPTICON 2 system (MJ research, MA, USA) and Go Taq Flexi DNA polymerase (Promega, WI, USA) used for PCR reactions as described previously [33,34]. The sequences of primers used in this study are 5′-TGAAGGTCAGACTGAGGCACC-3′ and 5′-AATTGGAAATGGTTTCCGTCC-3′ for *Uch-L3*, 5′-GATAACGAGATGCCACCAGAGG-3′ and 5′-TCCACGTCGGTTCTGTTCTTC-3′ for *Alp*, 5′-TGCACCCAGATCCTATAGCC-3′ and 5′-CTCCATCGTCATCATCATCG-3′ for *Opn*, 5′-GAGTCTGACAAAGCCTTCA-3′ and 5′-AGCCATACTGGTCTGATAG-3′ for *Oc*, 5′-ACAGCGGGCGAGGTGGTACTTG-3′ and 5′-TCCGGTGGTCCCGACTTCAGA-3′ for *ID1*, 5′-GCCCAGCACCCAGC GACAA-3′ and 5′-CGGCACCTCCAGCCGGTC-3′ for *Noggin*, and 5′-GGCATTGCTCTCAATGACAA-3′ and 5′-TGTGAGGGAGATGCTCAGTG-3′ for *Gapdh*.

2.3. Recombinant retrovirus production and transduction into the cells

Uch-L3 was overexpressed in C2C12 cells by retroviral transduction using previously described method [35]. Briefly, the HEK 293 cells were co-transfected with the retroviral Uch-L3 vector, pGag, and pVSV, and then incubated for 48 h. The viral supernatant were collected and incubated in C2C12 cells. The cells were selected 24 h after the transduction by puromycin (4 μg/ml) for 5 days.

2.4. siRNA-mediated knock-down of Uch-L3

The mouse Uch-L3 siRNA and scrambled siRNA (negative control) were purchased from Bioneer Co. (Daejeon, Korea). The duplex was transiently transfected into C2C12 cells with HiPerfect transfection reagent (Qiagen, CA, USA) according to the manufacture's protocol. Briefly, C2C12 cells were seeded on six-well plates. Each siRNA (200 nM) was transfected and incubated for 24 h, and then the media was changed before the treatment of BMP2 (100 ng/ml, jPC, Incheon, Korea). The cells were collected after 24 h for RNA isolation.

2.5. Co-immunoprecipitation

The cells were lysed with a buffer containing 10 mM Tris, 200 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA, 10% glycerol, and a protease inhibitor cocktail (Roche, Mannheim, Germany) [36]. The lysates were immunoprecipitated with either anti-Flag antibody or anti-HA antibody, and the eluents were loaded on a 12% SDS–PAGE gel. The blots were incubated overnight at 4 °C with either anti-HA antibody or anti-Flag antibody. After washing in TBS containing 0.05% Tween 20, the blots were incubated for 1 h at room temperature with anti-mouse IgG horseradish peroxidase-linked secondary antibody. The protein bands were visualized by ECL (Amersharm Pharmacia Biotech, NJ, USA).

2.6. In vivo ubiquitination assay

The HEK 293 cells were transiently transfected with the plasmids, pcDNA-HA/Flag-Smad1, pcDNA-Flag/HA-Ub, and/or pcDNA-Flag-Uch-L3. After 16 h, the transfected cells were treated with the proteasome inhibitor MG-132 (10 μM) for an additional 8 h. The cell lysates were immunoprecipitated with anti-HA antibody overnight at 4 °C. After 3 times washing, the eluents were immunoblotted against anti-Flag or Smad1 antibodies.

2.7. Alkaline phosphatase (ALP) staining

ALP staining was performed as previously reported [37]. Briefly, osteogenic transdifferentiation was induced in C2C12 cells for 24 h with BMP2 stimulation. The cells were then washed with 1× PBS, fixed with 2% PFA, and stained for alkaline phosphatase.

2.8. Immunocytochemistry

The C2C12 cells were seeded on cover glass in six-well plate. The mouse Uch-L3 siRNA and scrambled siRNA (negative control) were transfected. After 24 h of transfection, the cells were treated with BMP2 (100 ng/ml) for 3 h and fixed in 4% paraformaldehyde solution for 1 h. After the fixation, the cells were incubated in the freezer in the presence of ice-cold 100% EtOH. After the blocking with 3% BSA, the anti-phospho Smad1 antibody (1:500) was incubated overnight at 4 °C. Then, the cells were incubated in the anti-rabbit alexa-Fluor-555-conjugated secondary antibody (1:500) in the dark. The counterstaining was performed with DAPI for visualization of nuclei (Vector Lab., Burlingame, CA). The 1× PBS was used in the washing steps (3 times) between steps. The photography was taken by a fluorescent microscope (Zeiss, Exton, PA).

3. Results

3.1. The deubiquitinating enzyme Uch-L3 physically interacted with Smad1

Ubiquitin C-terminal hydrolase L3 (Uch-L3) was found in our previous proteomic study as a possible modulator sufficient to identify the specific targets in bone morphogenetic protein (BMP) 2-induced osteoblast differentiation [27]. To investigate the interaction between Uch-L3 and Smad1, HA-Smad1 and Flag-Uch-L3 plasmids were transfected into HEK 293 cells. Co-immunoprecipitation (IP) was performed against HA-Smad1 using anti-HA antibody. Uch-L3 protein (Flag-Uch-L3) was detected in the eluent of the immuno-complex by western blot using anti-Flag antibody (Fig. 1A). Reciprocally, the co-IP against Flag-Uch-L3 demonstrated that the eluent contained HA-Smad1 (Fig. 1B). Furthermore, it wondered whether the endogenous Smad1 interacts with Uch-L3. We determined the endogenous Uch-L3 levels in primary

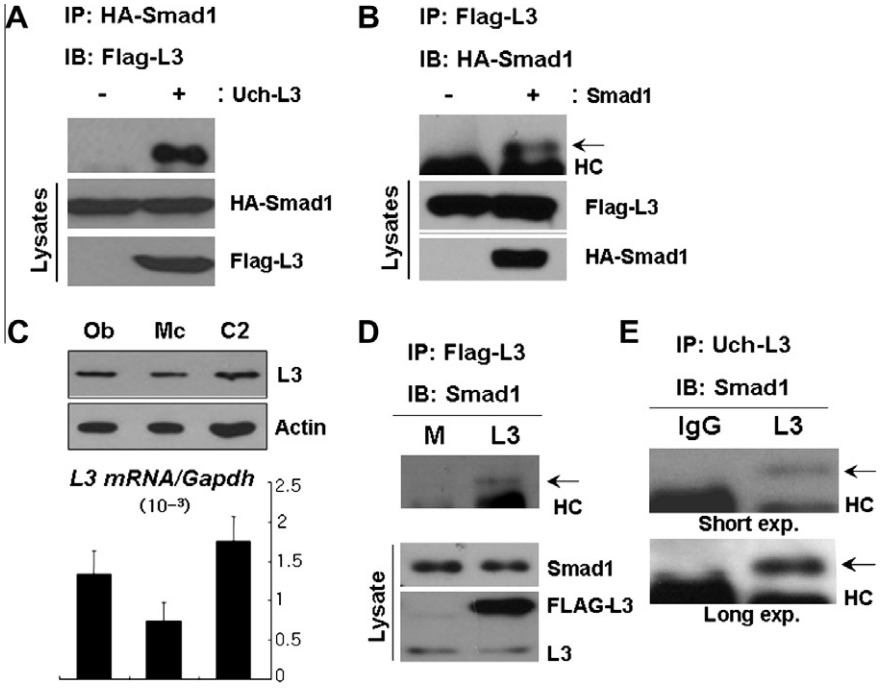

Fig. 1. The physical interaction between Uch-L3 and Smad1. The expression vectors for HA-Smad1 and/or Flag-Uch-L3 (Flag-Uch-L3 and/or HA-Smad1) were transfected into HEK293 cells. After 24 h, the transfectants were lysed and immunoprecipitated. (A) Co-immunoprecipitation (Co-IP) against HA-Smad1 using anti-HA antibody was performed and the eluent was immunoblotted against Flag-Uch-L3 using anti-Flag antibody. (B) Co-IP against Flag-Uch-L3 using anti-Flag antibody was performed and the eluent was immunoblotted against HA-Smad1 using anti-HA antibody. (C) Ten micrograms proteins of cell lysates were loaded on a 12% SDS–PAGE gel. The blot was incubated with anti-Uch-L3 antibody or anti-β-actin antibody. Isolated RNAs from the cells were used for real-time RT-PCR analysis with Uch-L3 primers. The values of Uch-L3 mRNA were normalized by *Gapdh*. Ob, primary osteoblast cells; Mc, MC3T3-E1 cells; C2, C2C12 cells. (D) The stable Uch-L3-C2C12 cells were established by retroviral transduction of Uch-L3 (L3) or by mock transduction (M). Co-IP against Flag-HA-Uch-L3 using anti-Flag antibody was performed with the lysate from the stable cells and the eluents were immunoblotted against anti-Smad1 antibody. (E) Co-IP against the endogenous Uch-L3 using anti-Uch-L3 antibody was conducted with the lysate from C2C12 cells and the eluent was immunoblotted against anti-Smad1 antibody. A normal rabbit IgG was used as a negative control for immunoprecipitation. The arrow indicates the Smad1 band. HC indicates heavy chain of IgG, a non-specific band. Short exp.; short exposure, Long exp.; long exposure.

osteoblast (Ob), MC3T3-E1 (Mc), and C2C12 (C2) cells. In those cells, similar levels of Uch-L3 proteins (Fig. 1C, upper panels) and mRNA levels (Fig. 1C, lower panels) were observed. Among them, C2C12 cells were selected to make the Uch-L3 stable cells. As shown in Fig. 1D, the endogenous Smad1 was also detected in the immunoprecipitated complex of Uch-L3 (Flag-Uch-L3). Furthermore, the endogenous Uch-L3 was also interacted with the endogenous Smad1 in C2C12 cells as confirmed by co-IP against anti-Uch-L3 antibody and WB using anti-Smad1 antibody (Fig. 1E). All of these results showed that Uch-L3 interacts with Smad1.

3.2. The poly-ubiquitinated Smad1 was dramatically decreased by Uch-L3

After confirming the interaction between Smad1 and Uch-L3, we tested whether the deubiquitinating activity of Uch-L3 is able to affect the ubiquitination pattern of Smad1. After the transfection of HA-Smad1 and Flag-Ub constructs with increasing amount of Uch-L3 or without Uch-L3 plasmid into HEK 293 cells, the degree of Smad1 ubiquitination was analyzed. The addition of Uch-L3 construct showed the significant deubiquitination of Smad1 by a dose-dependent manner (Fig. 2A, left panel). In the presence of Uch-L3, the amount of poly-ubiquitinated Smad1 was dramatically diminished compared to the control (in the absence of Uch-L3). Reciprocally, poly-ubiquitinated Smad1 was also detected in the HA-labeled ubiquitin/ubiquitinated protein complex immunoprecipitated with HA-Ub by anti-HA antibody. In addition, the amount of poly-ubiquitinated Smad1 was also decreased in the presence of Uch-L3 (Fig. 2B, top panel). These results suggest that Uch-L3 can deubiquitinate the poly-ubiquitinated Smad1.

3.3. Uch-L3 enhances the differentiation of osteoblast

We then tested whether Uch-L3, a possible DUB enzyme of Smad1, is able to accelerate the differentiation of osteoblast. The efficient stable expression of Uch-L3 was produced by the retroviral transduction onto C2C12 cells. The Uch-L3-expressing stable cells were selected by puromycin for 5 days. After the selection, we confirmed the expression of exogenous Flag-HA-Uch-L3 from the stable cell by Western blot (Fig. 3A). The activity of alkaline phosphatase (ALP), a downstream molecule in Smad1 pathway, was clearly enhanced as detected by ALP staining in Uch-L3 over-expressing cells compared to that in mock control cells (Fig. 3B, upper panels). The enhanced ALP activity may be caused by the deubiquitinating activity of Uch-L3 stabilizing Smad1. Moreover, BMP2-induced stimulation further increased ALP activity in Uch-L3 over-expressing cells compared to control cells (Fig. 3B, lower panels).

It is known that BMP2 activates Smad1 and induces the expression of the downstream genes. We checked the expression of some downstream target genes: alkaline phosphatase (*Alp*), osteopontin (*Opn*), osteocalcin (*Oc*), *ID1*, and *Noggin*. The induction of these genes was confirmed by real-time RT-PCR with BMP2 treatment (100 ng/ml). The expressions of *Opn*, *Alp*, *Oc*, *ID1*, and *Noggin* were higher in Uch-L3 stably-expressing cells compared to control cells (Fig. 3C). These results of increased expression of differentiation markers and down-stream target genes suggest that Uch-L3 may

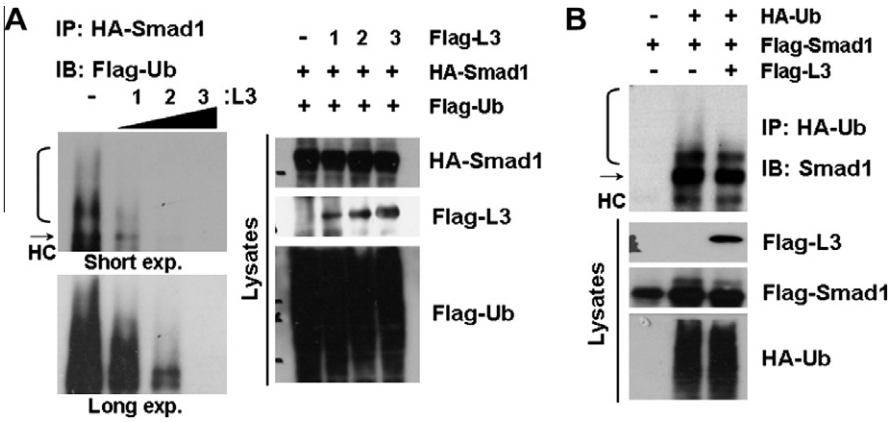

Fig. 2. The reduction of ubiquitinated Smad1 by Uch-L3. The expression vectors for HA-Smad1 and Flag-Ubiquitin (Flag-Ub), or HA-Ubiquitin (HA-Ub) and Flag-Smad1 with/without Flag-Uch-L3 were transfected into HEK293 cells. (A) The transfectants were lysed and incubated with anti-HA antibody for in vivo HA-Smad1 ubiquitination assay. The eluent was immunoblotted with anti-Flag antibody. The numbers 1, 2, and 3 indicate the amount of Flag-Uch-L3 plasmid in μg, which was transfected into the cells. (B) The lysates of transfectants were incubated with anti-HA antibody to capture HA-Ub conjugated proteins for in vivo ubiquitination assay. The eluent was immunoblotted with anti-Smad1 antibody. Total lysates were also immunoblotted with anti-Flag antibody for the detection of Uch-L3 and Smad1. '[' indicates the poly-ubiquitinated proteins. Arrow indicates mono-ubiquitinated Smad1. HC indicates heavy chain of IgG.

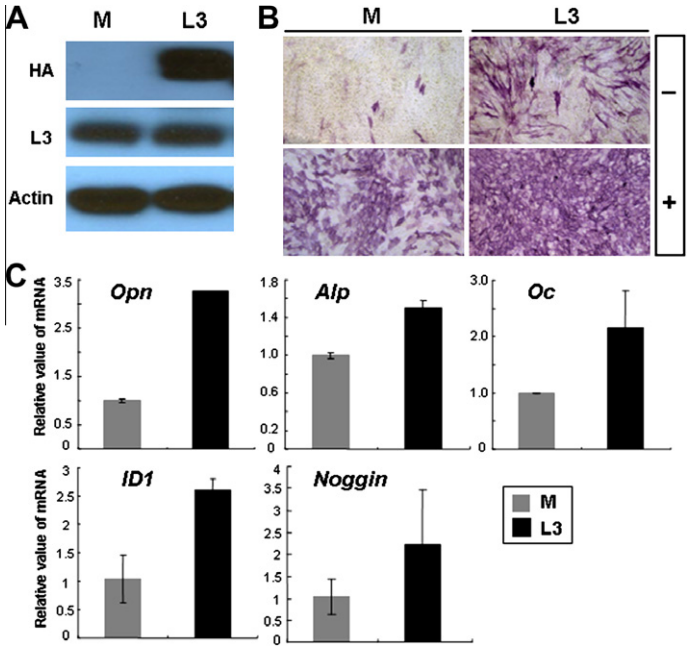

Fig. 3. The effect of Uch-L3 over-expression on osteoblast differentiation. The stable C2C12 cells were established by retroviral transduction of Uch-L3 (L3) or by mock transduction (M). (A) The C2C12 cells were lysed and immunoblotted with the indicated antibodies. (B) The stable C2C12 cells were treated with (+) or without (−) BMP2 (100 ng/ml) for 1 day. Alkaline phosphatase staining was performed on the cells. (C) The isolated RNA from the stable cells was reverse-transcribed and real-time PCR was conducted with the primers for *Opn*, *Alp*, *Oc*, *ID1*, and *Noggin*. The relative values of each mRNA were normalized by *Gapdh*. The data are expressed as mean ± S.D. of three different experiments. M, Mock control cells; L3, Uch-L3 stable cells.

enhance osteoblast differentiation by elevating the stability of Smad1.

3.4. Delayed osteoblast differentiation by the reduction of Uch-L3

To determine the effect of Uch-L3 knock-down in osteoblast differentiation, Uch-L3 specific siRNA was transfected into C2C12 cells. The expressions of Uch-L3 mRNA and protein after transfection were evaluated by real-time RT-PCR and immunoblotting (Fig. 4A). We then checked the osteoblast differentiation markers in the cells transfected with the Uch-L3 specific siRNA and the scrambled siRNA (negative control). The ALP staining was greatly reduced in Uch-L3 knock-down cells compared to the scrambled siRNA control cells (Fig. 4B). The mRNA expression levels of the markers, *Opn*, *Alp*, and *Oc* were also reduced in the Uch-L3 siRNA cells compared to the control cells (Fig. 4C). Furthermore, in the Uch-L3 knock-down cells transfected with L3-siRNA, the level of BMP2-induced phosphorylated Smad1 (pSmad1) was dramatically reduced 3 h after BMP2 treatment (Fig. 4D). In the immunocytochemical staining, the Uch-L3 knock-down cells showed the reduced expression of pSmad1 in the nucleus 3 h after BMP2 treatment, while the cells transfected with scrambled siRNA (Ctl)

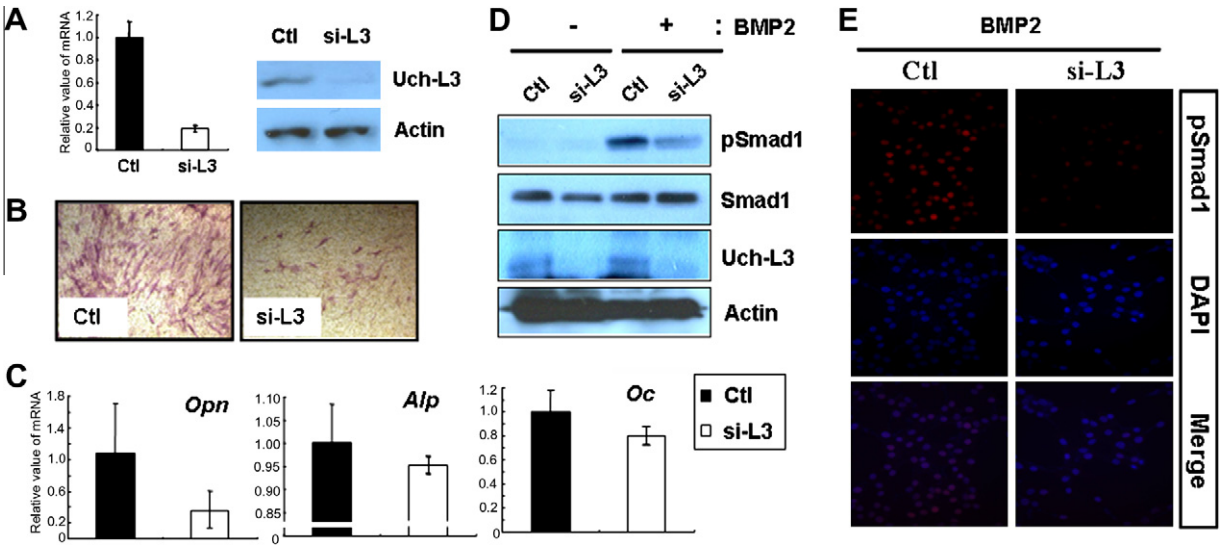

Fig. 4. The effect of Uch-L3 knock-down on osteoblast differentiation. (A) The scrambled siRNA and Uch-L3 siRNA duplexes were transfected into C2C12 cells. The isolated RNA from those cells was reverse-transcribed and the level of Uch-L3 expression was determined by real-time PCR (left panel). The cells were lysed and immunoblotted with anti-Uch-L3 antibody (right panel). (B) The C2C12 cells transfected with scrambled siRNA or Uch-L3 siRNA for 24 h. The cells were treated with BMP2 (100 ng/ml) for 1 day and stained by the alkaline phosphatase. (C) Real-time PCR was conducted using reverse-transcribed RNA and the primers of *Opn*, *Alp*, and *Oc* in the C2C12 cells transfected with scrambled siRNA or Uch-L3 siRNA and treated with BMP2 (100 ng/ml) for 1 day. The relative values of each mRNA were normalized by *Gapdh*. The data are expressed as mean ± S.D. of three different experiments. (D) After BMP2 treatment (100 ng/ml, 3 h) in the C2C12 cells transfected with either scrambled siRNA or Uch-L3 siRNA, the lysates were immunoblotted against anti-pSmad1, Smad1, or Uch-L3 antibodies. β-Actin was used as a loading control. (E) The C2C12 cells transfected with either scrambled siRNA or Uch-L3 siRNA were immunostained with anti-pSmad1 antibody. The cell nuclei were stained with a DAPI as a counterstaining. The fluorescent images were captured by a fluorescent microscopy (magnification, 400×). Ctl, the scrambled siRNA; si-L3, Uch-L3 specific siRNA.

showed strong pSmad1 expression in the nucleus (Fig. 4E). These results suggest that the reduction of Uch-L3 decreases the active pSmad1 signal and thus reduces the osteoblast differentiation.

4. Discussion

This study showed a novel function for Uch-L3 in osteoblast differentiation induced by the regulation of BMP2 signaling. The physical interaction between Uch-L3 and Smad1 was revealed. The activity of deubiquitinating enzyme Uch-L3 dramatically reduced the amount of ubiquitinated Smad1. In Uch-L3 stably-expressing cells, the expression of osteoblast differentiation markers, specifically osteocalcin, osteopontin, and alkaline phosphatase, was increased. In the Uch-l3 knock-down experiment, loss of Uch-L3 resulted in reduced ALP staining and the reduced expression of the markers. These results suggest that Uch-L3 plays a role as a modulator in the BMP2-mediated osteoblastogenesis and thus enhancing the differentiation of osteoblasts.

In our previous study, we reported elevated phosphorylation of Uch-L3 in BMP2-induced osteoblast differentiation [27]. The loss of Uch-L3 affects the accumulation of ubiquitinated proteins and the induction of stress [23]. A defect of Uch-L3 also resulted in retinal degeneration and neurodegeneration [6,18]. The up-regulation of Uch-L3 expression was observed in breast invasive cancer and cervical carcinoma [25,26]. Recent in vivo studies showed that the Uch-L3 deficiency reduced insulin signaling and adipogenesis [22]. However, there is no report on the possibility of Uch-L3 regulating osteoblast differentiation.

It has been reported that two proteins, Nedd8 and caseinolytic peptidase B (CLPB), can interact with Uch-L3 [32,38]. The C-terminal of Nedd8, an ubiquitin-like protein, is cleaved by Uch-L3. The CLPB, a protein in disaggregating machines, was revealed as a partner protein of Uch-L3 from a global proteomic approach. Inhibiting Uch-L3 activity caused increased ubiquitination in epithelial sodium channel (ENaC), which is regulated by the ubiquitin E3 ligase Nedd4-2 [39]. Although these studies highlight many important functions of Uch-L3, there is little known about the role of Uch-L3 in BMP signaling.

In TGFβ signaling, two known deubiquitinating enzymes, Usp9X (FAM) or Uch-L5 (Uch37) are known to act on the stability of the target proteins, Smad4 or Smad7 and type I TGFβ receptor, respectively [12,13]. Recently, it was reported that Uch-L1 positively regulates the stability of β-Catenin in Wnt signaling [14]. As those reports, UCH family members Uch-L1 and Uch-L5 which have 31–55% identity, function as deubiquitinating enzyme. However, there is no information on the involvement of deubiquitinating enzymes in BMP signaling and/or the differentiation of osteoblasts. In this study, it was found Smad1 is physically interacted with Uch-L3, which deubiquitinates Smad1. That is, Smad1 poly-ubiquitination degrees were decreased by the forced expression of Uch-L3. Furthermore, the forced expression of Uch-L3 promoted the osteoblast differentiation, and Uch-L3 knock-down showed reduced differentiation.

This study shows a novel finding that osteoblast differentiation could be regulated by Uch-L3, a deubiquitinating enzyme, through the BMP2-induced Smad1 stabilization. Future studies using the Uch-L3 knock-out mouse model will be necessary to understand the in vivo functions of Uch-L3.

Acknowledgments

This research was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2010-0020553 & No. 2010-0011231) and partly by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare (No. A090610 & No. A010252).

References

[1] Shanmugham, A. and Ovaa, H. (2008) DUBs and disease: activity assays for inhibitor development. Curr. Opin. Drug Discov. Dev. 11, 688–696.
[2] Singhal, S., Taylor, M.C. and Baker, R.T. (2008) Deubiquitylating enzymes and disease. BMC Biochem. 9 (Suppl. 1), S3.

[3] Sorokin, A.V., Kim, E.R. and Ovchinnikov, L.P. (2009) Proteasome system of protein degradation and processing. Biochemistry 74, 1411–1442.
[4] Bhoj, V.G. and Chen, Z.J. (2009) Ubiquitylation in innate and adaptive immunity. Nature 458, 430–437.
[5] Wilkinson, K.D. (2009) DUBs at a glance. J. Cell Sci. 122, 2325–2329.
[6] Kurihara, L.J., Kikuchi, T., Wada, K. and Tilghman, S.M. (2001) Loss of Uch-L1 and Uch-L3 leads to neurodegeneration, posterior paralysis and dysphagia. Hum. Mol. Genet. 10, 1963–1970.
[7] Jin, W., Chang, M., Paul, E.M., Babu, G., Lee, A.J., Reiley, W., Wright, A., Zhang, M., You, J. and Sun, S.C. (2008) Deubiquitinating enzyme CYLD negatively regulates RANK signaling and osteoclastogenesis in mice. J. Clin. Invest. 118, 1858–1866.
[8] Petroski, M.D. (2008) The ubiquitin system, disease, and drug discovery. BMC Biochem. 9 (Suppl. 1), S7.
[9] Nijman, S.M., Luna-Vargas, M.P., Velds, A., Brummelkamp, T.R., Dirac, A.M., Sixma, T.K. and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes. Cell 123, 773–786.
[10] Kurihara, L.J., Semenova, E., Levorse, J.M. and Tilghman, S.M. (2000) Expression and functional analysis of Uch-L3 during mouse development. Mol. Cell. Biol. 20, 2498–2504.
[11] Senta, H., Park, H., Bergeron, E., Drevelle, O., Fong, D., Leblanc, E., Cabana, F., Roux, S., Grenier, G. and Faucheux, N. (2009) Cell responses to bone morphogenetic proteins and peptides derived from them: biomedical applications and limitations. Cytokine Growth Factor Rev. 20, 213–222.
[12] Dupont, S., Mamidi, A., Cordenonsi, M., Montagner, M., Zacchigna, L., Adorno, M., Martello, G., Stinchfield, M.J., Soligo, S., Morsut, L., Inui, M., Moro, S., Modena, N., Argenton, F., Newfeld, S.J. and Piccolo, S. (2009) FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell 136, 123–135.
[13] Wicks, S.J., Haros, K., Maillard, M., Song, L., Cohen, R.E., Dijke, P.T. and Chantry, A. (2005) The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling. Oncogene 24, 8080–8084.
[14] Bheda, A., Yue, W., Gullapalli, A., Whitehurst, C., Liu, R., Pagano, J.S. and Shackelford, J. (2009) Positive reciprocal regulation of ubiquitin C-terminal hydrolase L1 and beta-catenin/TCF signaling. PLoS ONE 4, e5955.
[15] Datto, M. and Wang, X.F. (2005) Ubiquitin-mediated degradation a mechanism for fine-tuning TGF-beta signaling. Cell 121, 2–4.
[16] Gao, M. and Karin, M. (2005) Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. Mol. Cell 19, 581–593.
[17] Wicks, S.J., Grocott, T., Haros, K., Maillard, M., Ten Dijke, P. and Chantry, A. (2006) Reversible ubiquitination regulates the Smad/TGF-beta signalling pathway. Biochem. Soc. Trans. 34, 761–763.
[18] Sano, Y., Furuta, A., Setsuie, R., Kikuchi, H., Wang, Y.L., Sakurai, M., Kwon, J., Noda, M. and Wada, K. (2006) Photoreceptor cell apoptosis in the retinal degeneration of Uchl3-deficient mice. Am. J. Pathol. 169, 132–141.
[19] Semenova, E., Wang, X., Jablonski, M.M., Levorse, J. and Tilghman, S.M. (2003) An engineered 800 kilobase deletion of Uchl3 and Lmo7 on mouse chromosome 14 causes defects in viability, postnatal growth and degeneration of muscle and retina. Hum. Mol. Genet. 12, 1301–1312.
[20] Kwon, J. (2007) The new function of two ubiquitin C-terminal hydrolase isozymes as reciprocal modulators of germ cell apoptosis. Exp. Anim. 56, 71–77.
[21] Setsuie, R., Suzuki, M., Kabuta, T., Fujita, H., Miura, S., Ichihara, N., Yamada, D., Wang, Y.L., Ezaki, O., Suzuki, Y. and Wada, K. (2009) Ubiquitin C-terminal hydrolase-L3-knockout mice are resistant to diet-induced obesity and show increased activation of AMP-activated protein kinase in skeletal muscle. FASEB J. 23 (12), 4148–4157.
[22] Suzuki, M., Setsuie, R. and Wada, K. (2009) Ubiquitin carboxyl-terminal hydrolase l3 promotes insulin signaling and adipogenesis. Endocrinology 150, 5230–5239.
[23] Setsuie, R., Suzuki, M., Tsuchiya, Y. and Wada, K. (2010) Skeletal muscles of Uchl3 knockout mice show polyubiquitinated protein accumulation and stress responses. Neurochem. Int. 56, 911–918.
[24] Fang, Y., Fu, D. and Shen, X.Z. (2010) The potential role of ubiquitin c-terminal hydrolases in oncogenesis. Biochim. Biophys. Acta 1806, 1–6.
[25] Miyoshi, Y., Nakayama, S., Torikoshi, Y., Tanaka, S., Ishihara, H., Taguchi, T., Tamaki, Y. and Noguchi, S. (2006) High expression of ubiquitin carboxy-terminal hydrolase-L1 and -L3 mRNA predicts early recurrence in patients with invasive breast cancer. Cancer Sci. 97, 523–529.
[26] Rolen, U., Kobzeva, V., Gasparjan, N., Ovaa, H., Winberg, G., Kisseljov, F. and Masucci, M.G. (2006) Activity profiling of deubiquitinating enzymes in cervical carcinoma biopsies and cell lines. Mol. Carcinog. 45, 260–269.
[27] Kim, B.G., Lee, J.H., Ahn, J.M., Park, S.K., Cho, J.H., Hwang, D., Yoo, J.S., Yates III, J.R., Ryoo, H.M. and Cho, J.Y. (2009) 'Two-stage double-technique hybrid (TSDTH)' identification strategy for the analysis of BMP2-induced transdifferentiation of premyoblast C2C12 cells to osteoblast. J. Proteome Res. 8, 4441–4454.
[28] Ryoo, H.M., van Wijnen, A.J., Stein, J.L., Lian, J.B. and Stein, G.S. (1997) Detection of a proliferation specific gene during development of the osteoblast phenotype by mRNA differential display. J. Cell. Biochem. 64, 106–116.
[29] Kim, H.J., Kim, J.H., Bae, S.C., Choi, J.Y., Kim, H.J. and Ryoo, H.M. (2003) The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. J. Biol. Chem. 278, 319–326.
[30] Bakker, A. and Klein-Nulend, J. (2003) Osteoblast isolation from murine calvariae and long bones. Methods Mol. Med. 80, 19–28.
[31] Mayack, S.R. and Wagers, A.J. (2008) Osteolineage niche cells initiate hematopoietic stem cell mobilization. Blood 112, 519–531.
[32] Sowa, M.E., Bennett, E.J., Gygi, S.P. and Harper, J.W. (2009) Defining the human deubiquitinating enzyme interaction landscape. Cell 138, 389–403.
[33] Liu, F., Hata, A., Baker, J.C., Doody, J., Harland, R.M. and Massague, J. (1996) A human mad protein acting as a BMP-regulated transcriptional activator. Nature 381, 620–623.
[34] Yoon, K.A., Chae, Y.M. and Cho, J.Y. (2009) FGF2 stimulates SDF-1 expression through the Erm transcription factor in Sertoli cells. J. Cell. Physiol. 220, 245–256.
[35] Heo, S.H., Choi, Y.J., Ryoo, H.M. and Cho, J.Y. (2010) Expression profiling of ETS and MMP factors in VEGF-activated endothelial cells: role of MMP-10 in VEGF-induced angiogenesis. J. Cell. Physiol. 224, 734–742.
[36] Kim, J.Y. and Ozato, K. (2009) The sequestosome 1/p62 attenuates cytokine gene expression in activated macrophages by inhibiting IFN regulatory factor 8 and TNF receptor-associated factor 6/NF-kappaB activity. J. Immunol. 182, 2131–2140.
[37] Kim, Y.J., Kim, B.G., Lee, S.J., Lee, H.K., Lee, S.H., Ryoo, H.M. and Cho, J.Y. (2007) The suppressive effect of myeloid Elf-1-like factor (MEF) in osteogenic differentiation. J. Cell. Physiol. 211, 253–260.
[38] Wada, H., Kito, K., Caskey, L.S., Yeh, E.T. and Kamitani, T. (1998) Cleavage of the C-terminus of NEDD8 by UCH-L3. Biochem. Biophys. Res. Commun. 251, 688–692.
[39] Butterworth, M.B., Edinger, R.S., Ovaa, H., Burg, D., Johnson, J.P. and Frizzell, R.A. (2007) The deubiquitinating enzyme UCH-L3 regulates the apical membrane recycling of the epithelial sodium channel. J. Biol. Chem. 282, 37885–37893.